Comparison and Analysis of ADH Isozymes From a Population of *Chrysoma pauciflosculosa*

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By

Andrea M. Winter

Biology

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Andrea M. Winter
Honors College Scholar

Date: 4/27/04

Bonnie Kelley, Ph. D.
Faculty Advisor

Date: 4/28/04

Carolyn R. Thompson, Ph. D.
Dean, University Honors College

Date: 5/08/04

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

COMPAPISON AND ANALYSIS OF ADH ISOZYMES FROM A POPULATION OF *CHRYSOMA PAUCIFLOSCULOSA*

By
Andrea M. Winter
Bachelor of Science
University of North Carolina at Pembroke

Chrysoma paucifloscula is a rare evergreen shrub that is located on a sandy ridge. There are two populations of Chrysoma located in Pembroke, North Carolina. No published information on isozyme studies or population genetics exists for this species. Isozymes are variable forms of an enzyme. Two enzymes, ADH and MDH, were chosen for the comparison. Two prior students at UNCP developed an initial protocol for studying isozymes in this species. There data was used for storage and grinding. The leaves were collected from one site near Purnell Swett High School in Pembroke, North Carolina. The leaves were homogenized, electrophoresed on acrylamide gels and stained for the particular enzyme being studied. It was determined that ADH was active in this population of Chrysoma.

Comparison and Analysis of ADH Isozymes From a Population of *Chrysoma pauciflosculosa*

INTRODUCTION

Chrysoma pauciflosculosa, commonly known as "Woody Goldenrod" is an evergreen shrub found along sandy ridges. This evergreen shrub was originally in the *Solidago* genus, but taxonomists created a new genus, *Chrysoma*. The species name remained *pauciflosculosa*. It is a small shrub that is 0.5 to 1.5 meters tall. It has branches with leaves that are narrowly oblong and 3-6 cm long. (Figure 1, p. 19) In late October, *Chrysoma pauciflosculosa* blooms with smooth heads of small yellow flowers.

Chrysoma pauciflosculosa is considered a rare plant species in North Carolina but is commonly found growing in the southeastern United States where there are coastal dunes, sand ridges, and hills. In Florida, Georgia, and Mississippi, *Chrysoma pauciflosculosa* is the most abundant. In South Carolina and North Carolina the plant is very localized, with only two counties having this species. There are two sites of *Chrysoma pauciflosculosa* found in Robeson County, NC approximately 1 mile apart. Both of the sites are sandy ridges and *Chrysoma* are scatted among oaks. (Figure 2, p.20)

Enzymes are biological catalysts. Enzymes help the cell carry out reactions or processes. The two enzymes that were chosen for comparison were ADH (Alcohol Dehydrogenase) and MDH (Malate Dehydrogenase). Variable forms of the same enzyme are called isozymes. Isozymes contain variations in the amino acid sequence of the same enzyme and they occur in plants in the same geographical locations. Isozymes are a powerful tool that is used to observe gene variability within and between populations of plants. Usually starch gels are used to separate molecules and the presence of separated

proteins is detected by enzymatic activity. Since the Oxendine Science Building is being renovated, the starch gel apparatus is in long-term storage and was unable to be located. The protocol was modified to use polyacraylmide gels.

Polyacrylamide gel electrophoresis (PAGE) is a technique used to determine the molecular weight of a protein. There are native PAGE and SDS PAGE gels. A native PAGE gel is used to separate on the basis of shape, charge, and size. SDS PAGE gels have a detergent that unfolds the protein and interacts with the unfolded protein to get a constant charge. SDS PAGE gels only separate based on molecular weight. Native PAGE gels were primarily used during this research since testing was for a functional protein.

There is no published information on isozyme studies or population genetics that exists for this particular species. However, two past biology students at the University of North Carolina at Pembroke had done some prior research on *Chrysoma pauciflosculosa.* Marie-Louise Locklear and Christopher Hayes Kennedy did some preliminary work on an initial protocol using starch gels. I used their data for grinding ratio and storage.

METHODOLOGY

Collection of samples

There were several methods of leaf collection that were used during this research. There are two sites of *Chrysoma pauciflosculosa* located in Robeson County, North Carolina. The site chosen is near Purnell Swett High School in Pembroke, North Carolina. Using gloves, tweezers, and scissors the leaves were collected in October of 2003. Six different plants were chosen and samples were taken. The plants chosen were

flowering and the leaves collected were in bundles towards the outside edge of the plant. The leaves appeared to be fresh and healthy. The leaves were placed in 50 ml Falcon tubes and were placed on ice. Back in the lab, the tubes containing the leaves were frozen in liquid nitrogen and placed in the –70°C freezer.

Preparing solutions

Once the samples were stored, buffers and reagents were prepared. The following solutions had to be prepared: 1.0 M Tris-HCl, 0.2 M Tris-HCl, 0.05 M Tris-HCl, 0.38 M Glycine, 1% NAD, 1% MTT, 1% PMS, and Grinding Buffer. Grinding Buffer was prepared using 0.01 M Tris-Hcl, 10 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, and 20% sucrose.

Once the solutions were made, gels were ready to be poured. Acrylamide 5.5% separator and 3% stacker gels were used. The apparatus was assembled by using 2 glass plates and separators. The gels were prepared by using 1.84 ml of 30% Acrylamide/Bis; 3.8 ml of 1.0 M Tris-HCL, pH 8.9; 4.32 ml of dH_20; 50 µl of 10% APS and 5 µl of TEMED for the separator. APS and TEMED were added just before pouring. After the separator solidifies, the stacker and the comb were added. The stacker was prepared by using 250 µl of 30% Acrylamide/Bis; 200 µl of 1.0 M Tris- HCL, pH 6.9; 2 ml of dH_20; 25 µl of 10% APS and 2.5 µl of TEMED. APS and TEMED were added just prior to use.

Grinding of plants

Before samples could be placed on the gel cell lysates had to be prepared. The leaves were removed from the freezer and thawed slowly. The leaves had been stored in the –70 ^{0}C freezer between 5 and 6 months. The leaves were removed from the stem and homogenized on ice in the grinding buffer. β-Mercaptoethanol (BME) was added to the

buffer just before grinding. Polyvinylpyrrolidone grains (PVP) were added to the plants just before grinding. Grinding was done by using a test tube and stirring rod. The amount of plant to buffer ratio was 1:2 g/ml. The plants were spun in a mid speed centrifuge at 1,300 rmp for 5 min and then for another 5 min to obtain a pellet and usable extract.

Electrophoresis/Staining

The gels were loaded with ADH, marker, and lysate. Other gels were run with ADH, marker, and MDH. The amount of concentrations varied. (Shown in tables 1, 2, and 3, p. 21-23) After the gels were loaded, they electrophoresed at 200 volts until the bands were near the bottom of the gel. The gels were removed from the electrophoresis chamber and stained for activity of either enzyme. ADH or MDH stain was added within 10 minutes after electrophoresis. ADH stain (29.7 ml) was prepared by using 25 ml of 0.2 M Tris HCL pH 7.0; 2.5 ml of 95% ethanol; 1 ml of 1% NAD; 0.5 ml of 1% MTT and 0.2 ml of 1% PMS. MDH stain (31.7 ml) was prepared by using 25 ml of 0.2 M Tris HCL pH 8.0; 5 ml of 2M DL Malate; 1 ml of 1% NAD; 0.5 ml of 1% MTT and 0.2 ml of 1% PMS. The gel was covered in stain and placed in an incubator at 37^0C, in the dark. The gel was incubated until color developed, approximately 5-10 minutes, then destained distilled water. Gel results were imaged by either a digital camera or a scanner, then the gels were stored in the dark.

Blot Test

A dot blot test was done to see if the reagents were working and to determine if commercial ADH and MDH were active. This was done by using different amounts of ADH, MDH, and grinding buffer. The amounts used were 1 µl of ADH mixed with 29 µl of grinding buffer, 3 µl of ADH mixed with 27 µl of grinding buffer, 10 µl of ADH

mixed with 20 µl of grinding buffer, and 30 µl of ADH with no buffer. The same amounts were used for MDH. 30 µl of ADH stain and MDH stain was added to the appropriate samples.

RESULTS

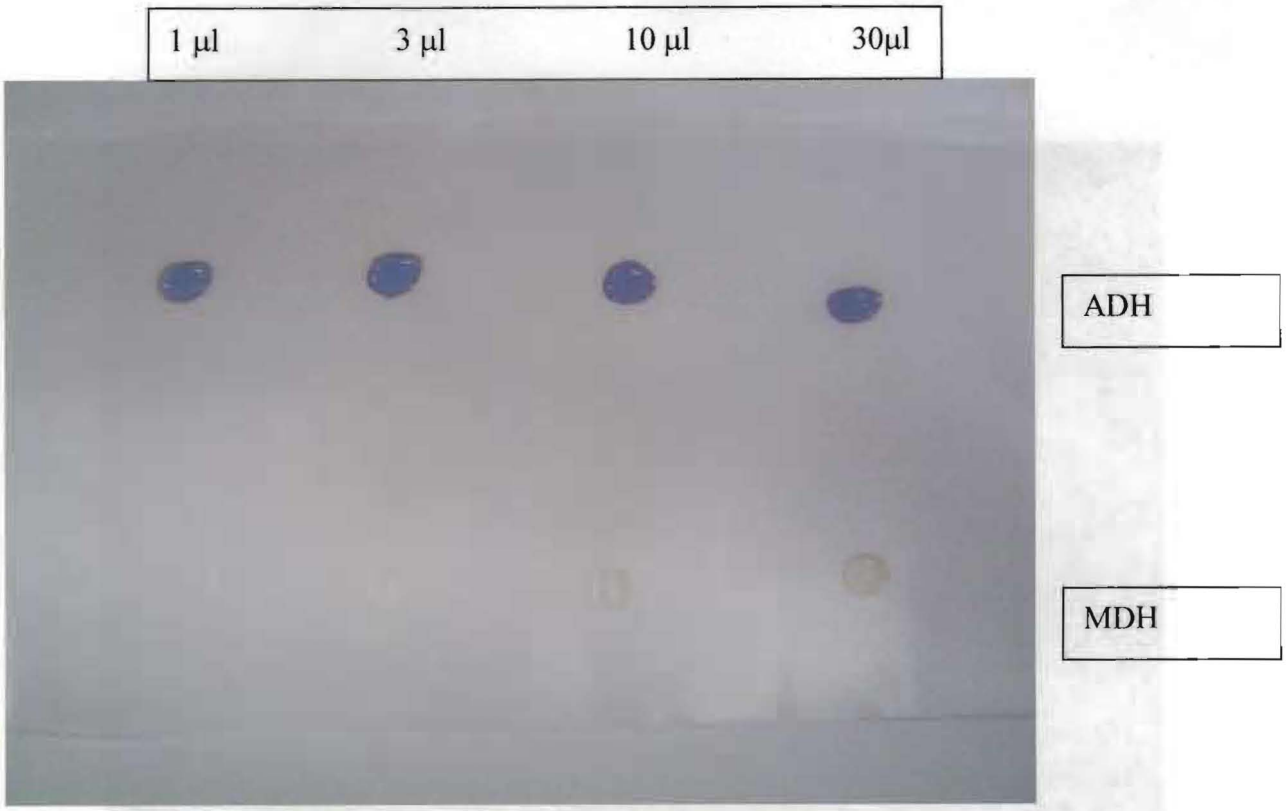
In preparing the first batch of gels, four native PAGE were made. The first gel did not have enough separator so it was unusable. The second gel leaked out. The third gel solidified correctly and was usable. In the fourth gel, the stacker was placed on too quickly and was unusable. Gel number three was used and a gel was run. Analysis of the gel showed no bands for ADH or MDH.

It was determined to do a blot test to see if the enzymes were active. ADH showed that the reagents were working. There was an immediate color change when the stain substrate was added. MDH showed no color change. This means that the commercial reagent is no longer good, probably due to the age of storage. There were no further gels stained with MDH because there was no positive control for that reagent.

Dot Blot Test

1 unit convert 1 μ mole of substrate

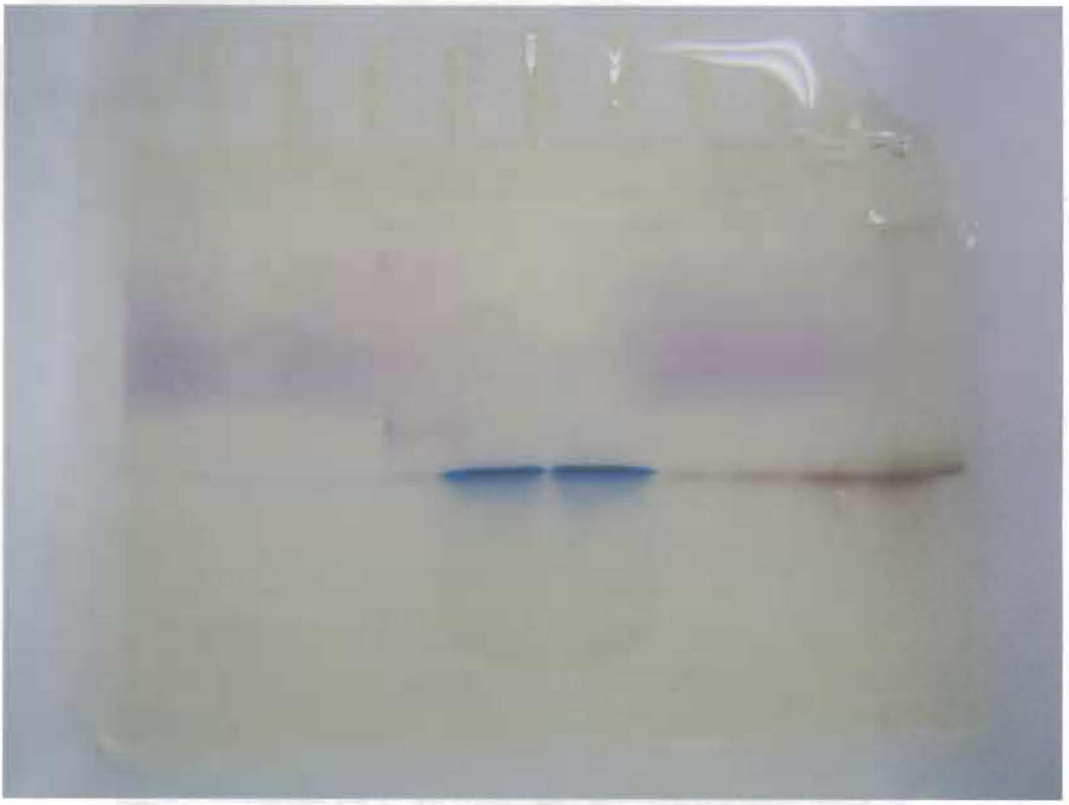
1 unit – 2 μg 30 units = 60 μg



ADH and MDH applied in variable concentrations (1-30 μl)

Another two gels were made and solidified correctly. One gel was run and there were bands that appeared on the ADH positive side. This proves that ADH is working and gave a control to compare the results from the plants. The side with the plants showed very faint smudges that indicate some ADH activity.

Gel with ADH, marker, and lysate

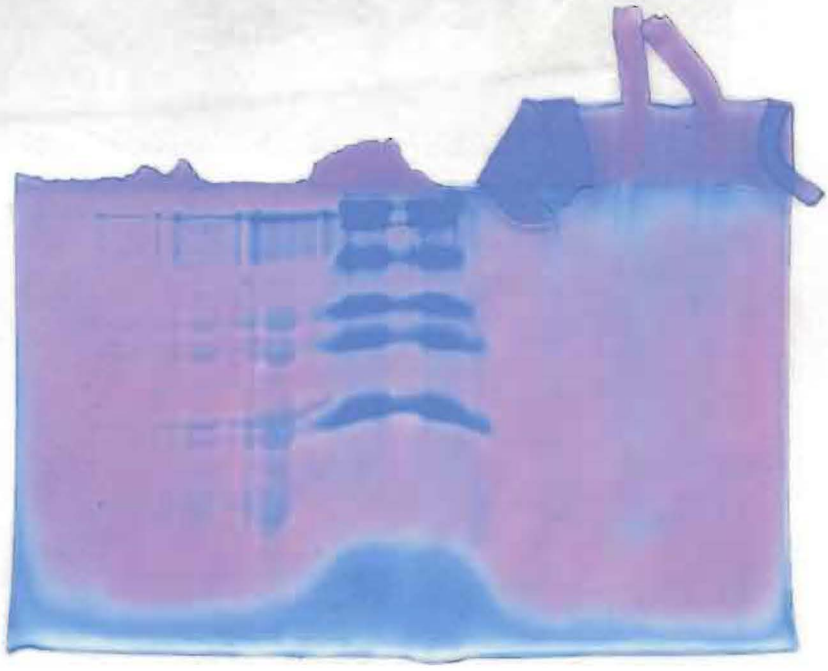


1 µl 3 µl 10µl 30µl Markers 4 µl 8µl 16µl 30µl

ADH Plants

A native PAGE gel and a SDS gel were made. ADH and plant A were run on both gels. The SDS PAGE showed bands for ADH but not the plants. This might have to do with the staining time. The stain was not left on the appropriate time, so bands may be there just not visible. The native page gel was scanned and the color was changed slightly so the purple was clearer. The molecular weight for ADH is around 40,000 -45,000 Da.

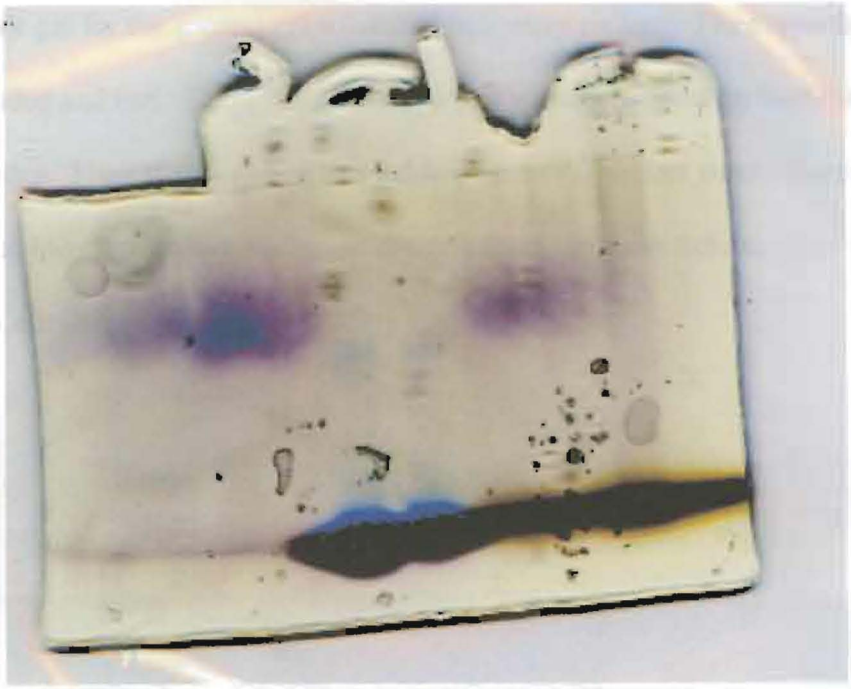
SDS PAGE Gel



1 µl 3 µl 10µl 30µl Markers 4 µl 8µl 16µl 30µl

ADH Plants

Native PAGE Gel



1µl 3µl 10µl 30µl Markers 4 µl 8µl 16µl 30µl

ADH Plants

Two more native PAGE gels were made. One solidified correctly, while the separator gel for the second gel solidified in a wavy pattern. The correctly solidified gel was loaded and run. There were smudges that were consistent on both the ADH and plant sides. Plant A was ran on this gel to compare from last week. The smudges were in the same location as the smudges in the gels from the week before. This shows that the plant A enzymes are similar.

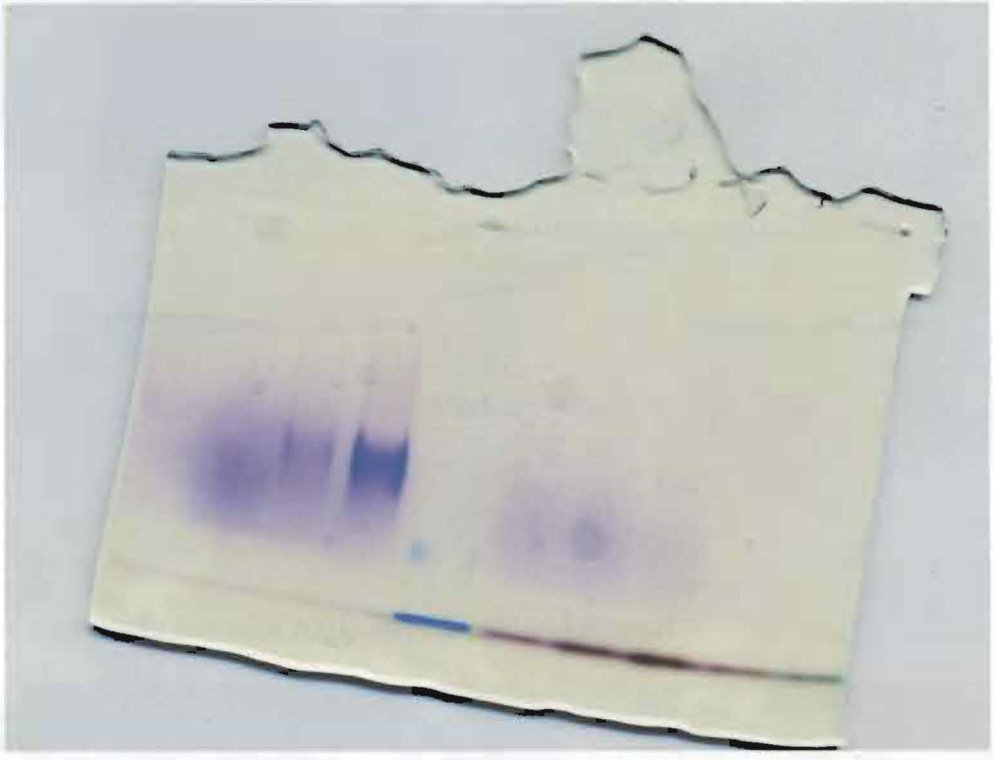
Native PAGE Gel with ADH and Plant A

1µl 3µl 10µl 30µl Marker 4 µl 8µl 16µl 30µl

ADH Plants

Two more gels were made and solidified correctly. ADH, marker, and samples from plant B were ran on one gel. ADH, marker and samples from plant C were ran on the other gel. Plant C was harder to retrieve an extract from. The lysate was stickier so 100 µl of grinding buffer was added. In the last lane of the gel, the plant material floated out of the top when it was loaded. More leaf lysate floated out of plant C than plant B.

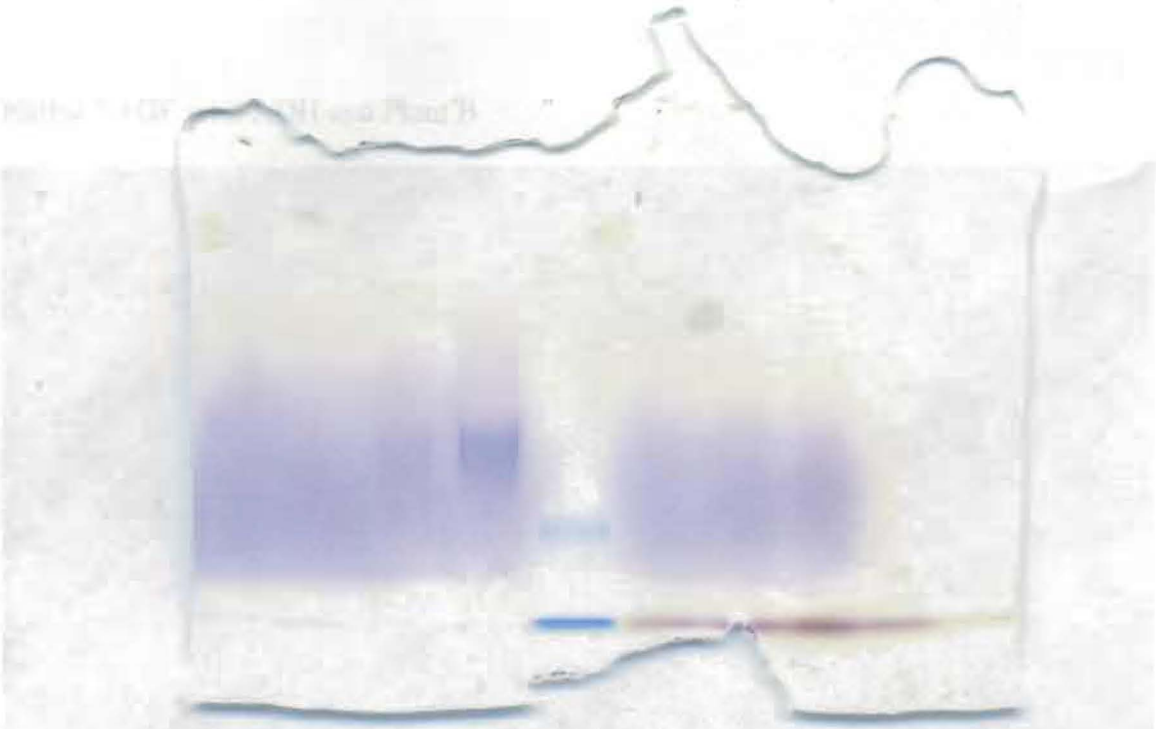
Native PAGE gel with ADH and Plant B



1µl 3µl 10µl 30µl Marker 4 µl 8µl 16µl 30µl

ADH Plants

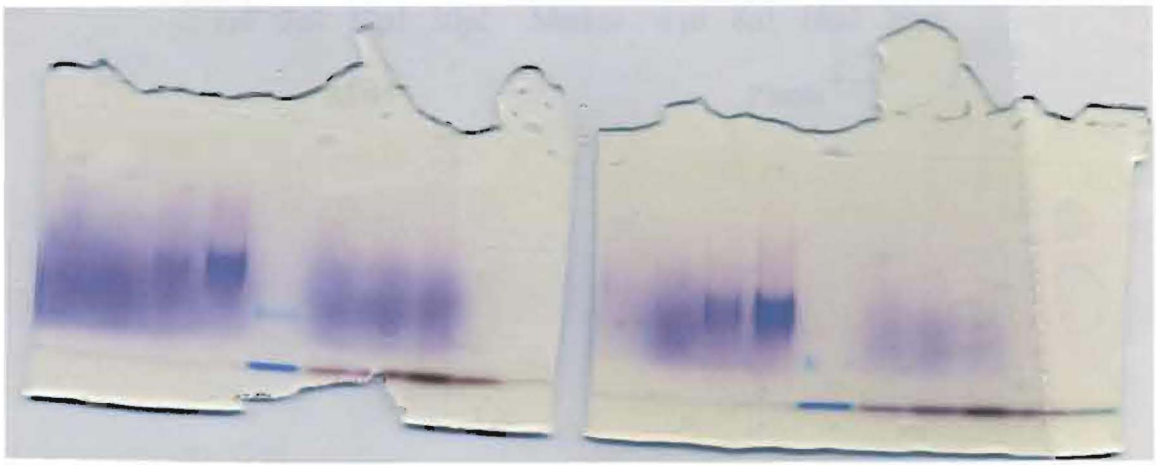
Gel with ADH and Plant C



1µl 3µl 10µl 30µl Marker 4 µl 8µl 16µl 30µl

ADH Plants

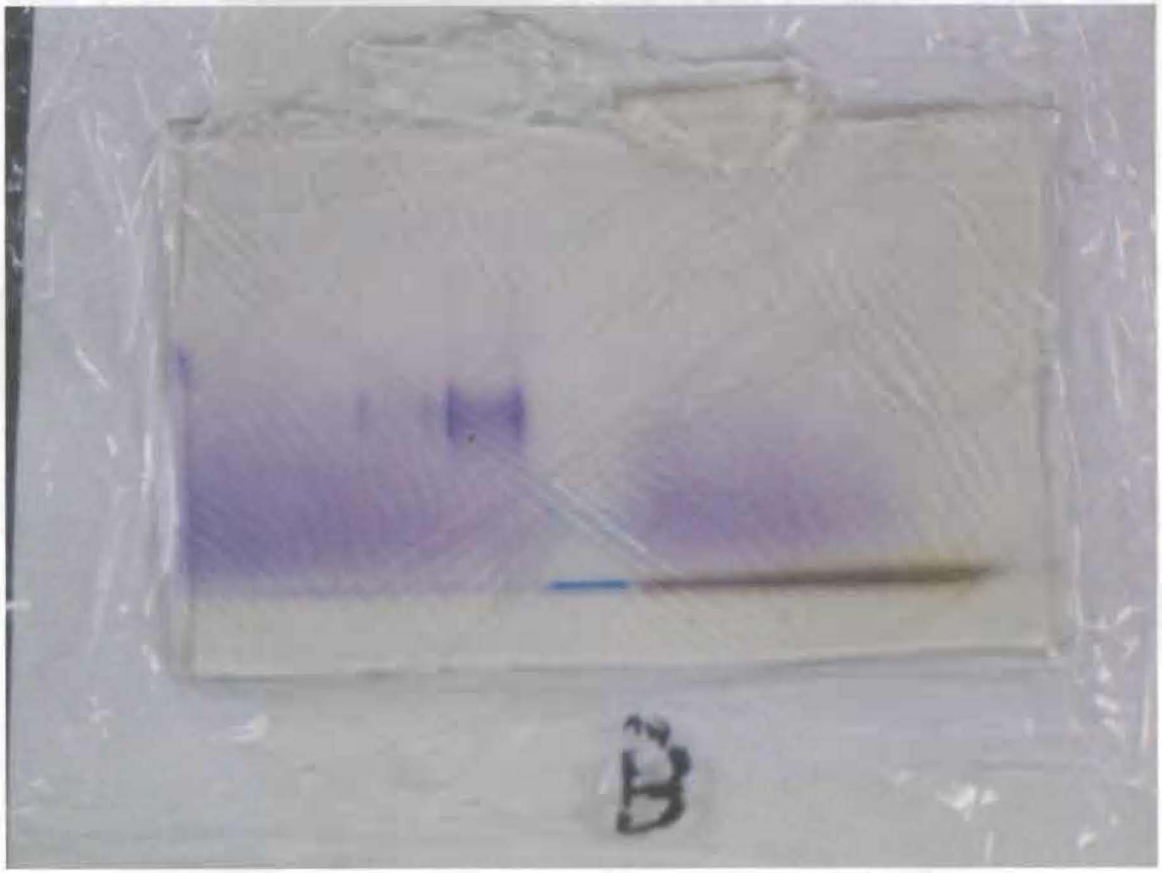
Comparing Gels with ADH and Plant C and Plant B



Plant C Plant B

Two more native PAGE gels were run with different grindings of plants B and C. This was done to repeat the procedure to be sure to get the same results.

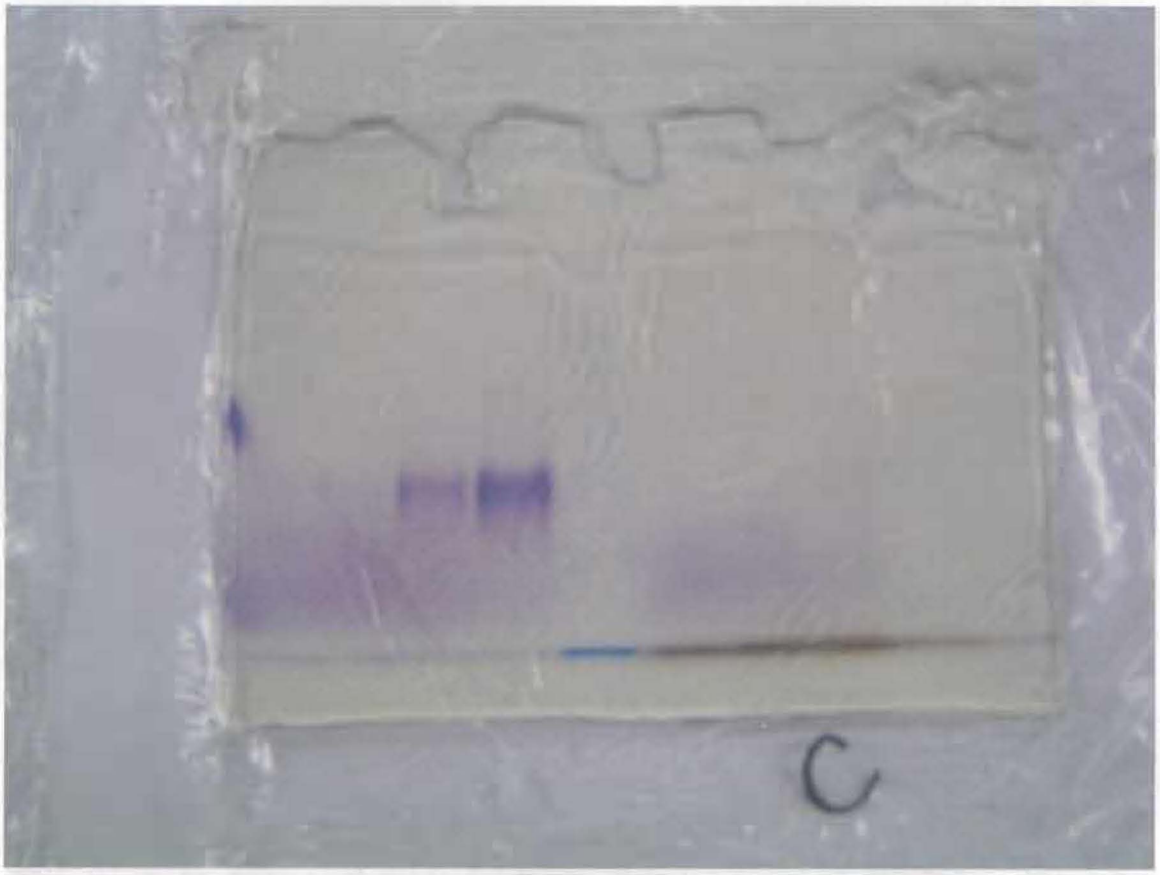
Native PAGE with ADH and Plant B



1µl 3µl 10µl 30µl Marker 4 µl 8µl 16µl 30µl

ADH Plants

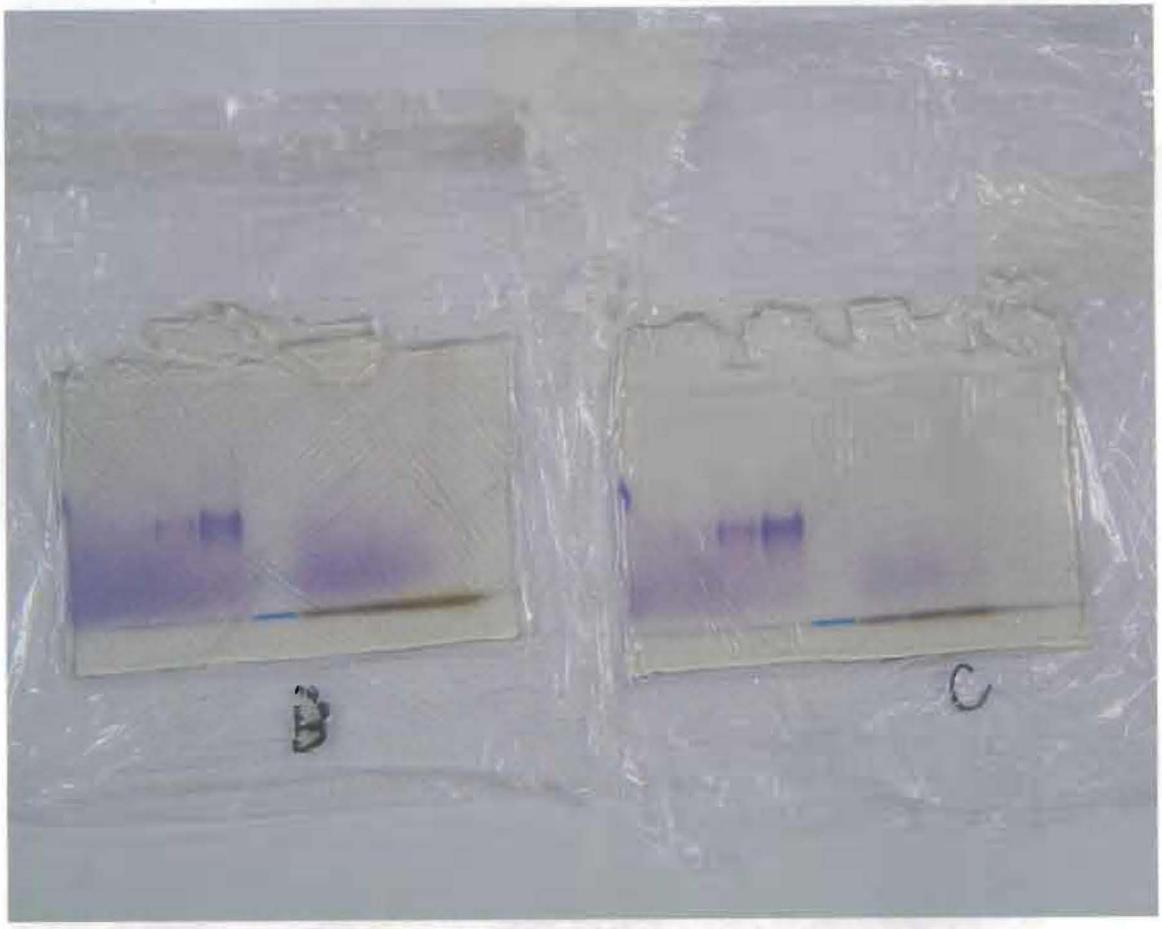
Native PAGE with ADH and Plant C



1µl 3µl 10µl 30µl Marker 4 µl 8µl 16µl 30µl

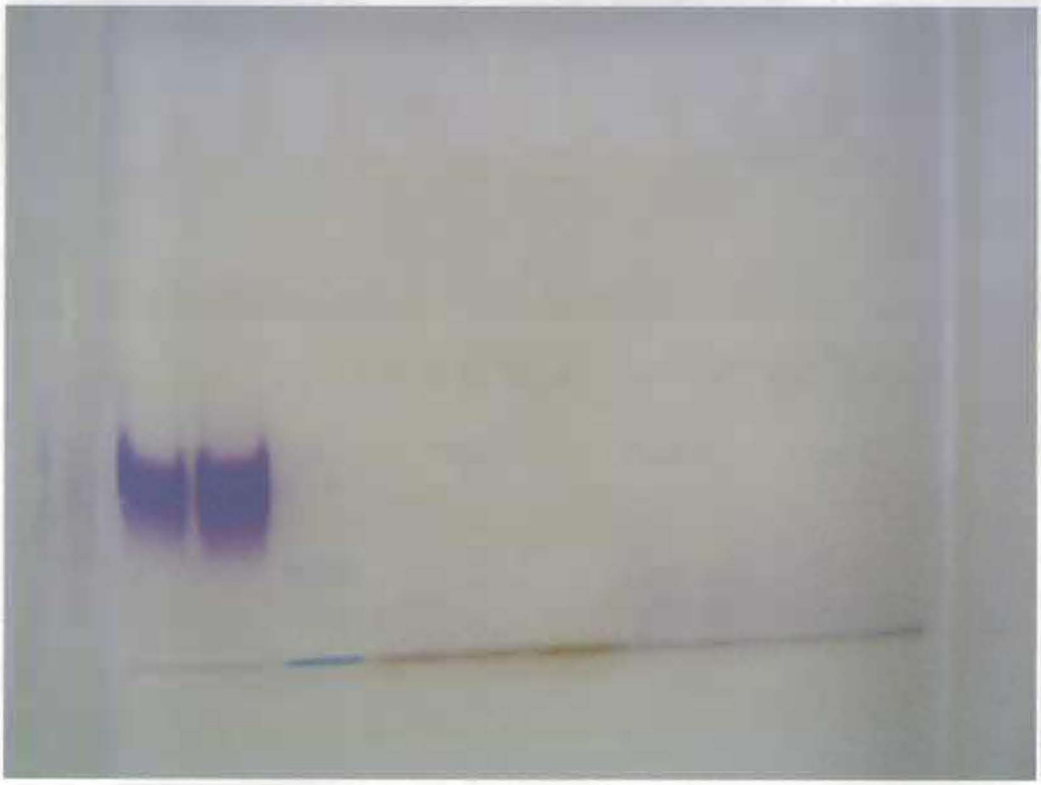
ADH Plants

Native PAGE with ADH, Plant B and Plant C



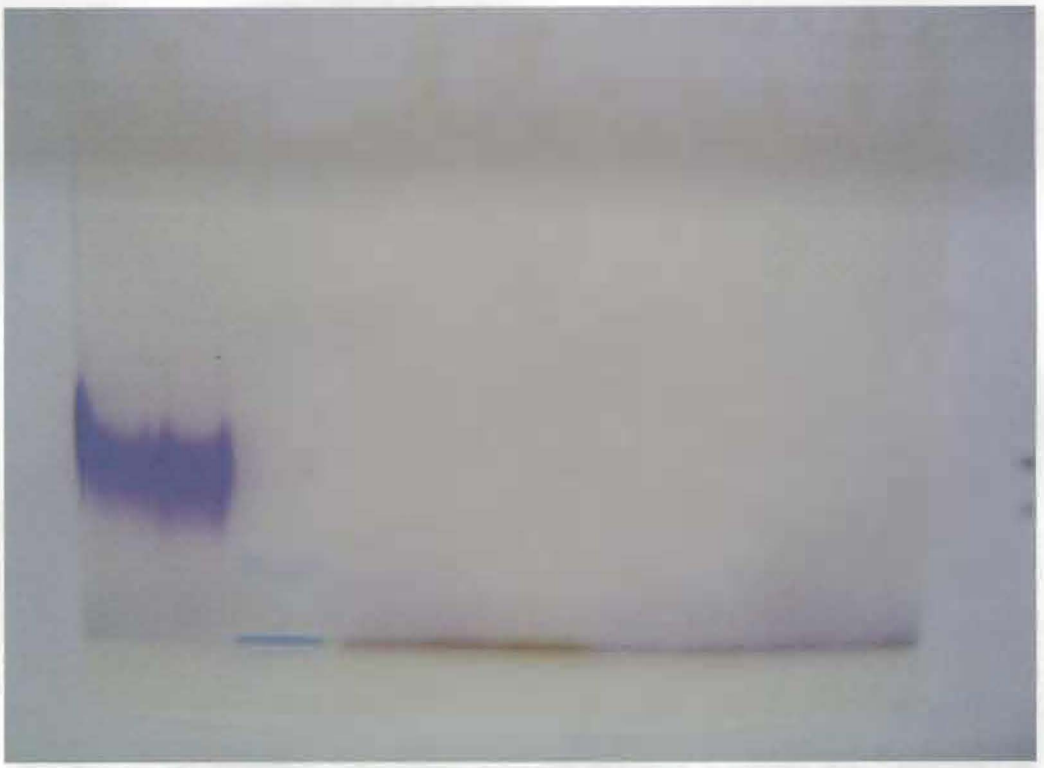
Two more native PAGE gels were made to compare plants A, B, and C. ADH activity showed up immediately when the stain was poured on the gels. The gels above indicate that ADH is forming aggregates each time it is frozen and thawed. To prevent this in the future, it would be advisable to sub-aliquot the stock ADH into smaller tubes and keep frozen until needed.

Plants A, B, C and ADH



10 µl	30 µl	5 µl	4 µl	8 µl	16 µl	4 µl	8 µl	4 µl	8µl
ADH		Marker		Plant A		Plant B		Plant C	

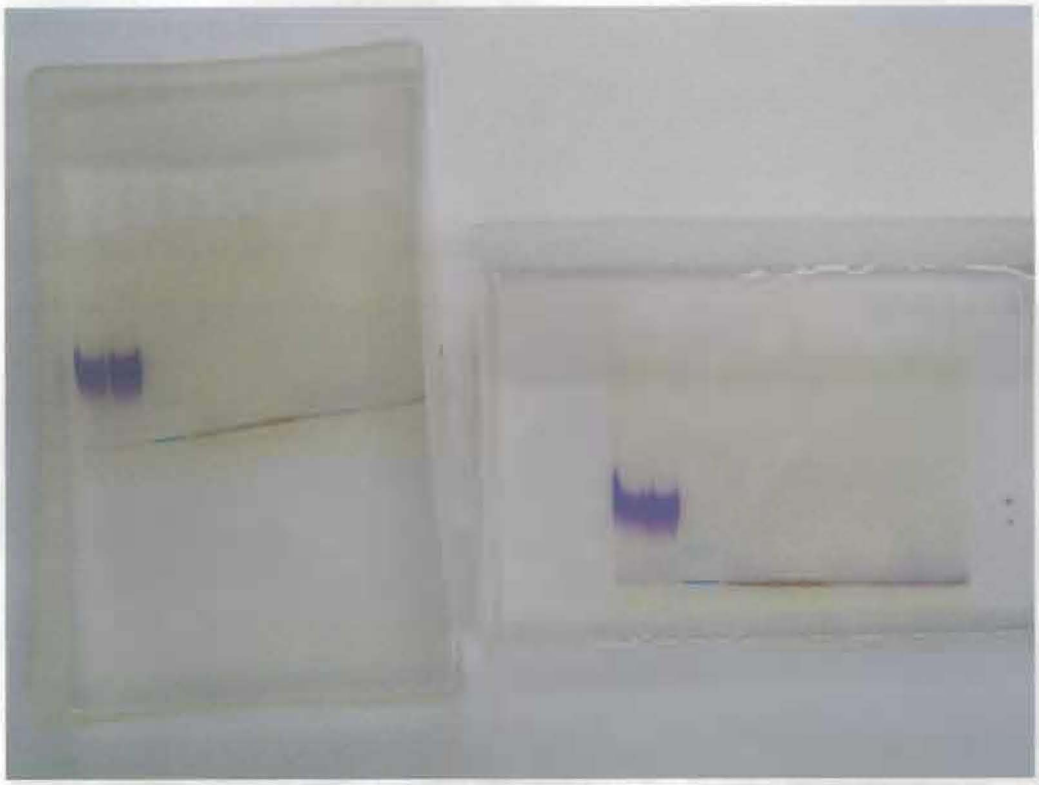
Plants A, B, C and ADH



10 µl 30 µl 5 µl 4 µl 8 µl 16 µl 4 µl 8 µl 4 µl 8µl

ADH Marker Plant A Plant B Plant C

Comparison of the two gels for plants A, B, and C



DISCUSSION

There is some ADH activity that is indicated on the gels. The larger the concentrations are on the gel, the less enzymatic activity observed. This indicates there is some type of inhibitor that is reacting with the ADH in the plants. The inhibitor is unknown at this time. Activity of the MDH could not be determined because the commercially derived enzyme (used for the positive control) was old. New enzyme was ordered but is on backorder until June. The samples were stored longer then the previous students had tested them to be stored. In the previous study, the leaves had been stored for 3 months. In this study the leaves were stored between 5 and 6 months. The samples

can be stored for over 5 months with enzyme activity identifiable. Being frozen and thawed each time ADH was used was aggregating the ADH. Sub aliquots of ADH would alleviate this problem.

Figure 1: *Chrysoma pauciflosculosa* in April

Figure 2: Sandy Ridge where *Chrysoma pauciflosculosa* is located

Table 1: Amounts of samples loaded on Native PAGE

Samples	Identity	Amount	Grinding Buffer
1	ADH (2 µg/µl) =1.26 units/µl	1 µl	29 µl
2	ADH	3 µl	27 µl
3	ADH	10 µl	20 µl
4	ADH	30 µl	-
5	Marker	10 µl	-
6	Marker	10 µl	-
7	Plant	4 µl	26 µl
8	Plant	8 µl	22 µl
9	Plant	16 µl	14 µl
10	Plant	30 µl	-

Table 2: Amount of ADH and MDH Loaded on Native PAGE

Samples	Identity	Amount	Grinding Buffer
1	ADH (2 µg/µl) =1.26 units/µl	1 µl	29 µl
2	ADH	3 µl	27 µl
3	ADH	10 µl	20 µl
4	ADH	30 µl	-
5	Marker	10 µl	-
6	Marker	10 µl	-
7	MDH	1 µl	29 µl
8	MDH	3 µl	27 µl
9	MDH	10 µl	20 µl
10	MDH	30 µl	-

Table 3: Amount of ADH and Plants Loaded on Native PAGE

Samples	Identity	Amount	Grinding Buffer
1	ADH	10 µl	20 µl
2	ADH	30 µl	-
3	Marker	5 µl	-
4	Plant A	4 µl	26 µl
5	Plant A	8 µl	22 µl
6	Plant A	16 µl	14 µl
7	Plant B	4 µl	26 µl
8	Plant B	8 µl	22 µl
9	Plant C	4 µl	26 µl
10	Plant C	8 µl	22 µl

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